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Exogenous leptin advances puberty in domestic hen

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Abstract

The present study was undertaken to examine the effect of recombinant chicken leptin administered to fed ad libitum and feed-restricted immature chickens of a layer strain on ovarian development and the timing of sexual maturity. In the first experiment 11-week-old pullets (77 days of age) fed ad libitum were injected daily with leptin at four dose levels (4, 16, 64 and 256 μg/kg body weight) until sexual maturity (lay of the first egg). Leptin treatment at the highest dose significantly ($P < 0.05$) advanced the onset of puberty (day 116.3 ± 1.0) in comparison to controls (day 121.3 ± 1.2). The rises of luteinizing hormone, estradiol and progesterone in blood plasma were also advanced by leptin treatment. In the second experiment, both full-fed and feed-restricted pullets (79 days of age) were injected daily with leptin (256 μg/kg body weight). In birds fed ad libitum, exogenous leptin again significantly ($P < 0.05$) advanced first ovipostion (day 118.4 ± 1.4 versus day 124.4 ± 1.7), while abolishing the significant ($P < 0.05$) delay caused by feed restriction (day 131.5 ± 1.6) and restoring the normal onset of sexual maturity (day 125.7 ± 1.6). Analysis of the ovaries in 106-day-old pullets revealed that leptin injections advanced follicular development, particularly in birds fed ad libitum, and significantly ($P < 0.01$) reduced follicular apoptosis both in full-fed and feed-restricted birds. In

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conclusion, we have shown that in female chickens exogenous leptin advances the onset of puberty by attenuation of ovarian apoptosis and enhancement of folliculogenesis.

Keywords: Leptin; Puberty; Chicken; Apoptosis; Ovary; Follicular development

1. Introduction

Since the discovery of leptin, a 146-amino acid protein [1] having a tertiary structure characteristic of cytokines [2] it has been revealed that leptin acts not only as a satiety, appetite-regulating hormone which controls weight gain and fat deposition (for review, see [3]), but that leptin is also extensively involved in reproductive processes and likely serves as a main hormonal factor that links adiposity with reproduction (for review, see [4,5]). The first indication of this association came from the observation that genetically obese mice lacking functional leptin (*ob*/*ob*) or leptin receptor (*db*/*db*) fail to undergo normal sexual maturation and remain infertile throughout life [6,7]. Similar findings have been also reported in humans (for review, see [8]). In *ob*/*ob* mice, recombinant leptin restores fertility by affecting reproductive function per se, not by simply reducing body weight [9,10]. In children with congenital leptin deficiency, administration of leptin may restore the gonadotrophic pulsatility characteristic of early puberty [11]. In normal prepubertal female mice and rats, leptin treatment accelerates the onset of puberty [12–14] or at least reverses the delay caused by food restriction [15–17]. In addition, transgenic female skinny mice overexpressing leptin by 12-fold show early signs of puberty [18] and mice overexpressing leptin by 5-fold undergo early puberty, suggesting a quantitative effect of leptin on the reproductive axis at puberty [19]. As reported most recently, leptin treatment is also effective in restoring ovulation and menstrual cycles in women with hypothalamic amenorrhea [20]. Furthermore, leptin seems to be important in mediating undernutrition-induced deficits in reproductive functions [21–23]. However, despite those numerous reports, the mechanism of leptin action has only been partially clarified.

Reports concerning the biological role of leptin in birds are scarce (for review, see [24,25]). The decrease in food intake observed in layer and broiler chickens injected centrally (i.c.v.) or peripherally (i.p., i.v.) with recombinant chicken, ovine or human leptin [26–29], and in a wild bird species injected (i.m.) with chicken leptin [30], suggests that in birds leptin may play a similar role in regulating the energy balance as it does in mammals. It has been also reported that recombinant leptin injected in ovo acts in Japanese quail as a growth factor accelerating embryonic and postembryonic development [31]. Leptin has a stimulatory effect on in vivo angiogenesis of embryo chorioalantoic membrane [32] and in vitro proliferation of embryonic muscle and liver cells [33] in chickens. Cloning of the chicken leptin receptor [34,35] and detection of its presence in the hypothalamus [34,36], the pituitary [36] and the ovary [35,36] indicate the possibility that leptin might be involved in the regulation of reproductive functions in birds by acting both at the central and peripheral levels. This possibility has been confirmed by our recent study showing that in mature laying chickens leptin injected during fasting ameliorates the negative effects of fasting on ovarian

function due to attenuation of follicular apoptosis and its stimulatory effect is mediated most likely by the expression of central as well as peripheral leptin receptors [36].

Our objective in the present study was to examine whether leptin may play a physiological role in the development of the avian ovary at sexual maturity. Immature female chickens, either fed ad libitum or feed-restricted, were injected with recombinant chicken leptin [26] prepared according to the cDNA sequence published by two independent groups [37,38] and exhibiting more than 80% similarity with most mamalian leptins [24]. Whether the recombinant protein used in our experiments is indeed the native chicken leptin [37–39] or is a mammalian-derived leptin analogue as suggested by others [40,41], remains matter of controversy. However, the recombinant protein produced in our laboratory exhibits leptin-like activities both in vitro and in vivo. These activities include: (i) activation of leptin receptor in Baf/3 cells stably transfected with the long form of human leptin receptor [26], (ii) recognition by antibodies raised against ovine leptin [42], (iii) interaction with the human [43] and chicken [44] leptin binding domain subcloned from the respective leptin receptors, (iv) attenuation of food intake in layer and broilers chickens [26,28,29] and finally (v) ability of attenuating the negative effects of fasting on ovarian function in mature layer chickens [36]. All these activities are likely transduced through leptin receptors. However, the recently published draft sequence of the chicken genome, which likely covers 90% of the genome [45], has revealed no sequence corresponding to that reported for the chicken leptin gene [37,38]. Further research is needed to resolve these uncertainties concerning the identity of the chicken leptin gene.

2. Materials and methods

2.1. Reagents

Recombinant chicken leptin was prepared by genetic engineering using prokaryotic expression vector pMON3401 encoding full size A (−1) chicken leptin [26]. Spectria estradiol [^{125}I] and progesterone [^{125}I] kits were purchased from Orion Diagnostica (Espoo, Finland) and in situ cell death detection kit (POD) from Roche Diagnostic (Mannheim, Germany). Purified chicken luteinizing hormone (LH) and rabbit antiserum against chicken LH were from USDA-ARS (Beltsville, MD, USA). All other chemicals were purchased from ICN Biomedicals (Aurora, IL, USA) or Sigma (St. Louis, MO, USA).

2.2. Animals and experimental designs

All animal experiments were conducted according to the research protocols approved by the State Committee for Scientific Research in Warsaw, Poland. Female chickens of layer strain (Isa Brown) were purchased at 7 week of age from the commercial farm Drobeco, Bielsko Biala, Poland. Birds were kept in individual cages and provided with commercial food and water ad libitum, under a photoperiod of 14 h light and 10 h dark with lights on at 07.00 h. Period of acclimation before starting experiments lasted about 4 weeks. Recombinant chicken leptin diluted PBS–BSA (1 mg/ml) was injected (s.c.) daily at (07.00–08.30 h) until the onset of puberty as evidenced by first oviposition. Control birds received PBS–BSA.

Feed intake and body weight were recorded daily. Blood was taken (07.00–8.30 h) from the wing vein and placed in heparinized tubes before leptin or PBS–BSA injection. After centrifugation (7 min, 1000 × *g*) plasma was collected and kept at −20 °C until luteinizing hormone (LH), estradiol (E2) and progesterone (P4) determinations.

2.3. Experiment 1

Eleven-week-old pullets (77 days of age; $n = 100$) were divided into five equal groups and injected with leptin at doses of 0 (control), 4, 16, 64 or 256 μg/kg body weight/day. Blood samples for determination of LH, E2 and P4 plasma concentrations were taken once a week for the first 4 weeks of the experiment and then two or three times a week. At the onset of puberty, the ovaries were isolated, weighed and characterized morphologically.

2.4. Experiment 2

Eleven-week-old pullets (79 days of age; $n = 96$) were divided into four equal groups: (1) fed ad libitum (control), (2) fed ad libitum + leptin, (3) feed-restricted and (4) feed-restricted + leptin. The food-restricted birds were fed 82–85% of the amount consumed by the control group. Leptin was injected at the dose of 256 μg/kg body weight/day. Blood samples for determination of E2 and P4 plasma concentrations were taken once a week for the first 4 weeks of the experiment and then two or three times a week. When pullets were 106 days of age, six birds were randomly chosen from each group, euthanized by decapitation and the ovaries were isolated, weighed and characterized morphologically. Paraffin sections of stromal tissue containing cortical follicles <1 and 1–4 mm white follicles were examined for histological and apoptotic changes. The onset of puberty for the other 18 birds from each group was recorded.

2.5. Hormonal assays

Plasma LH concentrations were determined using a homologous chicken LH radioimmunoassay. Purified chicken LH [46], LH antiserum and secondary antibody were provided by the USDA Animal Hormone Program, and the assay was performed according to recommendations supplied with the reagents. The lowest detectable concentration of LH was 100 pg and the intra- and interassay coefficients of variation were 3.1% and 6.2%, respectively. E2 and P4 concentrations in blood plasma were measured radioimmunologically using Spectria kits (Orion Diagnostica, Finland). The detection limit for E2 was 5.45 pg and for P4 was 90 pg. The intra- and interassay coefficients of variation for E2 and P4 were 5.9%, 6.2% and 4.4%, 5.1%, respectively.

2.6. Histology and apoptosis evaluation

The specified ovarian tissues were fixed in 4% (v/v) buffered paraformaldehyde, processed and embedded in paraffin wax. Sections (6 μm) were stained with haematoxyline and eosine and examined histologically using a Nicon-300 light microscope (Tokyo, Japan). For evaluation of apoptosis, deparaffinized sections were incubated with proteinase K

(20 μg/ml) in 10 mM Tris–HCl (pH 7.4, 37 °C, 20 min) and apoptotic cells were detected by the TdT-mediated dUTP nick-end labeling (TUNEL) method [47] using the in situ cell death detection kit (POD) according to manufacturer's instruction. Negative controls of the examined sections were incubated without terminal deoxynucleotidyl transferase. Sections were incubated with DAB–H_2O_2 mixture to visualize the immunoreaction products. TUNEL-positive cells were counted in the wall of stromal cortical follicles (<l mm) or white follicles (1–4 mm) on 10 randomly chosen fields (50 μm × 50 μm) of each examined tissue.

2.7. Statistical analysis

Statistical analysis was done using one- or two-way analysis of variance followed by Duncan's multiple range test. Results are expressed as mean ± S.E.M. and considered significantly different at $P < 0.05$.

3. Results

3.1. Experiment 1

Daily injections of leptin to immature female chickens resulted in a dose-related effect on the advancement of sexual maturation evidenced by the timing of first oviposition (Table 1). In comparison to the placebo-injected control birds, statistically significant ($P < 0.05$) acceleration of the onset of puberty was found in birds receiving the highest dose of leptin (256 μg/kg body weight/day). In parallel to the acceleration of sexual maturity, the rises of LH, E2 and P4 were also advanced by 2–6 days (Fig. 1). In the group that received the highest dose of leptin, the peak concentrations of LH, E2 and P4 occurred earlier than in the controls by 6, 4 and 5 days, respectively (Fig. 1B, D and F). Similarly, the highest levels of E2 in birds receiving 16 or 64 μg of leptin were observed 4 days earlier than in control birds or birds receiving 4 μg of leptin (Fig. 1C and D). The concentrations of P4 increased in all groups just prior to the first ovulation, beginning in all leptin-treated groups at an earlier age than in the controls (Fig. 1E and F).

Feed intake and body weight were monitored throughout the experiment. Feed consumption increased steadily with time, but there were no significant differences among the examined groups. During the first week of the experiment, average daily feed intake in

Table 1
The effect of recombinant chicken leptin on the onset of puberty in female chickens fed ad libitum

Leptin dose (μg/kg body weight/day)	First egg (day of age)	Body weight (g) at onset of puberty
0	121.3 ± 1.2 a	1578 ± 21 a
4	119.9 ± 1.1 a,b	1585 ± 21 a
16	118.6 ± 1.1 a,b	1595 ± 24 a
64	118.0 ± 0.9 a,b	1613 ± 23 a,b
256	116.3 ± 1.0 b	1672 ± 21 b

Each value is the mean ± S.E.M. from 20 birds. Means marked with different letters (a, b) are significantly different at $P < 0.05$.

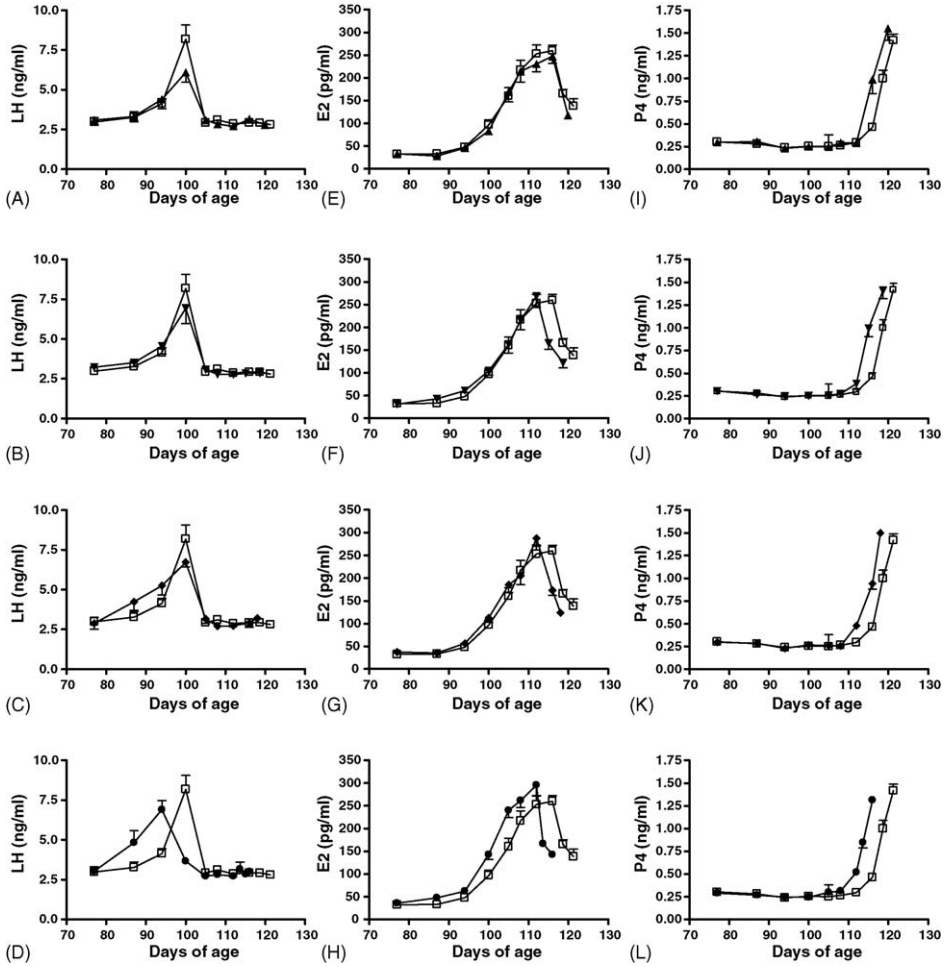

Fig. 1. The effect of increasing doses of recombinant chicken leptin on blood plasma concentrations of LH (A–D), estradiol (E2; E–H) and progesterone (P4; I–L) in immature female chickens fed ad libitum. Each value is the mean ± S.E.M. of 20 birds. To facilitate the comparison among treatment groups, the results are presented in four panels according to the leptin dose: control 0 μg leptin/kg body weight/day (□), 4 μg leptin/kg body weight/day (▲) (A, E and I), control 0 μg leptin/kg body weight/day (□), 16 μg leptin/kg body weight/day (▼) (B, F and J), control 0 μg leptin/kg body weight/day (□), 64 μg leptin/kg body weight/day (♦) (C, G and K) and control 0 μg leptin/kg body weight/day (□), 256 μg leptin/kg body weight/day (●) (D, H and L).

control group was 72.8 ± 1.20 g and in leptin-treated groups ranged from 73.2 ± 1.64 to 76 ± 1.75 g, while during the last week preceding the onset of puberty in control group was 94.8 ± 1.63 g and in leptin-treated groups ranged from 88 ± 3.61 to 98.4 ± 2.21 g. Body weight did not differ significantly among the examined groups through the first 3 weeks of leptin injections, but at 4 weeks and later, the body weight in the group receiving the highest dose of leptin (256 μg) was slightly higher as compared with placebo-treated control group

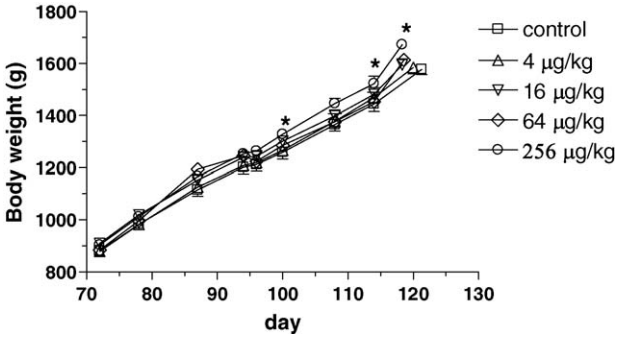

Fig. 2. The effect of increasing doses of recombinant chicken leptin on the body weight. At time points marked with asterisk (*) the differences between the control and 256 μg leptin/kg body weight/day treatment were statistically significant ($P < 0.05$).

(Fig. 2). At the onset of puberty, body weight was slightly (5.9%) but significantly ($P < 0.05$) higher in birds receiving 256 μg/kg body weight/day than in birds receiving 0, 4 or 16 μg of leptin (Table 1).

Morphological characteristics of the ovaries isolated just after first oviposition showed that at the onset of puberty the mean ovary weight of all groups was similar and there were no significant differences among the examined groups in the mean weight of ovarian stroma containing cortical follicles <1 mm as well as in the mean number and weight of white follicles (1–6 mm), yellowish follicles (>6–8 mm) and yellow hierarchical follicles (>8–28 mm, data not shown).

3.2. Experiment 2

The effect of daily injections of leptin on the onset of puberty in ad libitum-fed and feed-restricted immature female chickens is shown in Table 2. As in the first experiment, leptin administrated to ad libitum-fed birds significantly ($P < 0.05$) advanced the onset of puberty by 6 days. In feed-restricted birds, the first oviposition was significantly ($P < 0.05$) delayed by 7 days, but leptin treatment abolished this delay (Table 2). Feed restriction delayed the prepubertal rises of E2 (Fig. 3B versus Fig. 2A) and P4 (Fig. 3D versus Fig. 2C) in blood

Table 2
The effect of recombinant leptin on the onset of puberty in fed ad libitum and feed-restricted female chickens

Food restriction[a]	Leptin dose (μg/kg body weight/day)	First egg (day of age)	Body weight (g) at onset of puberty
No (control)	0	124.4 ± 1.7 b	1659 ± 21 a
No (control)	256	118.4 ± 1.4 a	1729 ± 17 b
Yes	0	131.5 ± 1.6 c	1575 ± 19 c
Yes	256	125.7 ± 1.4 b	1590 ± 15 c

Each value is the mean ± S.E.M. from 20 birds. Means marked with different letters (a, b, c) are significantly different at $P < 0.05$.

[a] 82–85% of ad libitum control group.

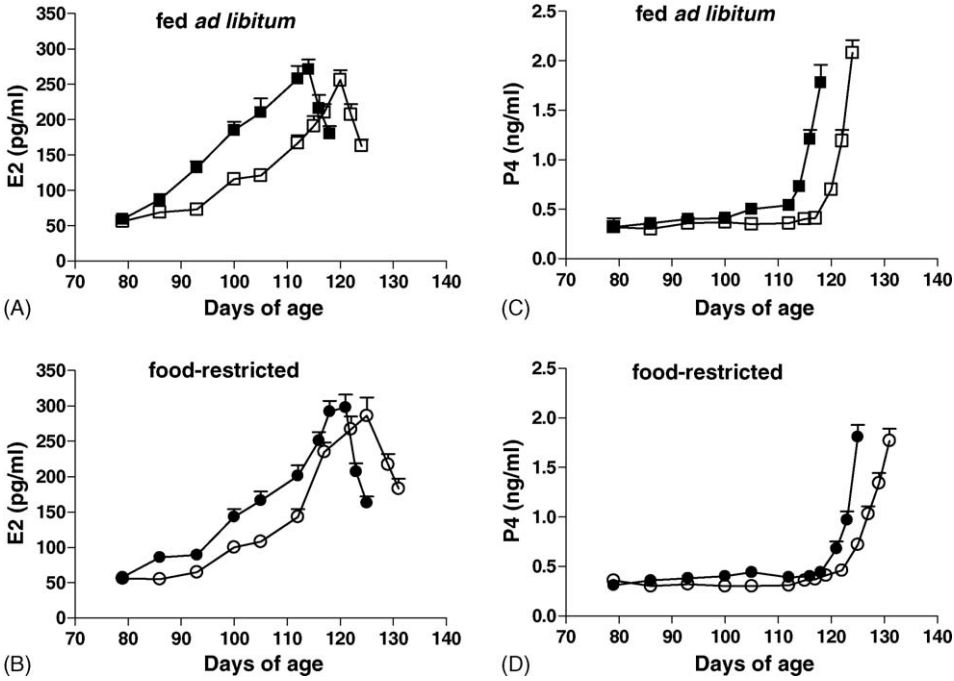

Fig. 3. The effect of recombinant chicken leptin on the blood plasma concentrations of estradiol (E2; A and B) and progesterone (P4; C and D) in immature female chickens fed ad libitum (A and C) and feed-restricted (B and D). Each value is the mean ± S.E.M. of 24 birds until day 106 and then from 18 birds. Treated (■, ●) or non-treated (□, ○) with leptin (256 μg/kg body weight/day). Food-restricted birds were fed 82–85% of ad libitum control group.

plasma, while leptin injections advanced the rises of these steroids in ad libitum-fed (Fig. 3A and C) as well as feed-restricted birds (Fig. 3B and D). Similarly as in the first experiment, in birds fed ad libitum, leptin treatment did not affect feed consumption but again slightly (4.2%) increased body weight at sexual maturity as compared to full-fed controls (Table 2). In contrast, leptin administration had no effect on body weight of feed-restricted pullets, where limited nutrients resulted in both groups being significantly smaller than full-fed controls (Table 2).

The effect of leptin treatment on the ovary weight in immature, 106-day-old chickens fed ad libitum and feed-restricted is presented in Fig. 4A. The ovaries of ad libitum birds injected with leptin were significantly ($P < 0.01$) larger, while those of feed-restricted birds were significantly ($P < 0.01$) smaller than ovaries of ad libitum-fed control birds. Leptin injections had no significant effect on the ovary weight in feed-restricted birds (Fig. 4A). The weight of ovarian stroma with cortical follicles <1 mm did not differ significantly among the groups when examined at 106 days of age (after receiving 27 daily injections), but the stage of follicular development differed among groups and was ordered as follows: ad libitum + leptin > ad libitum (control) > food-restricted + leptin > food-restricted (Table 3).

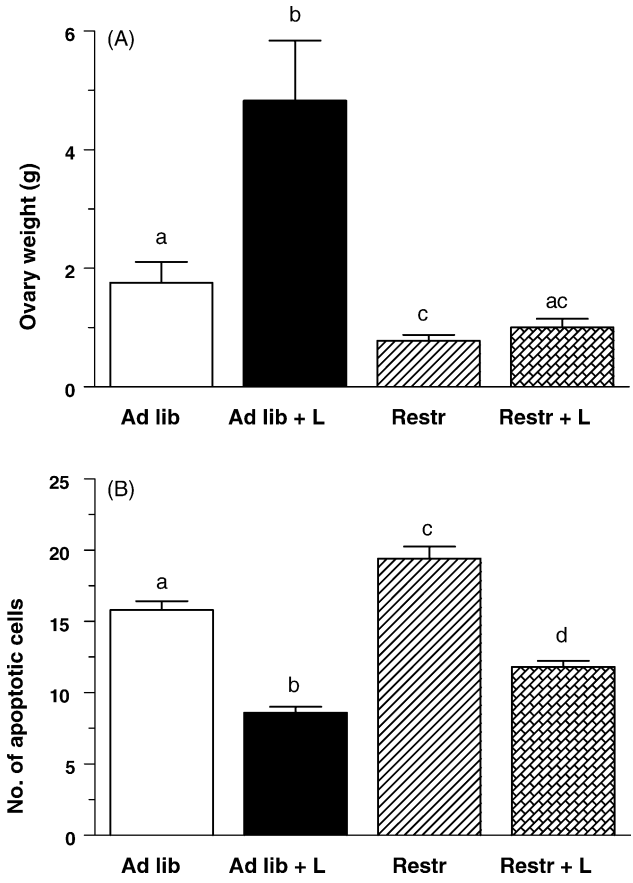

Fig. 4. The effect of recombinant chicken leptin on the ovary weight (A) and the number of apoptotic cells in the wall of stromal cortical follicles with a diameter <1 mm (B) in immature, 106-day-old chickens fed ad libitum and feed-restricted. Each value is the mean ± S.E.M. of six birds. Means marked with different letters are significantly different at $P < 0.01$. Leptin (L) was injected at the dose of 256 μg/kg body weight/day. Feed-restricted birds were fed 82–85% of ad libitum control group. Apoptotic cells (TUNEL-positive) counted on 10 randomly chosen fields (50 μm × 50 μm) of each stromal tissue were averaged for each bird for that microscopic field size and subsequently the mean value from six hens was calculated.

Histological examination of stromal tissue containing cortical follicles <1 and of 1–4 mm white follicles showed similar tissue structure in all examined groups. However, there were significant differences in the number of apoptotic cells in the wall of cortical follicles (Fig. 4B). Feed restriction per se increased the number of apoptotic cells in the wall of these follicles by 23% compared to full-fed counterparts ($P < 0.05$). Leptin treatment decreased the number of apoptotic cells in both feed-restricted and ad libitum-fed chickens by 39% and 46%, respectively ($P < 0.01$; Fig. 4B). Although a similar trend was observed in 1–4 mm white follicles, the effect of leptin was not significant (data not shown).

Table 3
The effect of recombinant chicken leptin on the ovary development in immature, 106-day-old female chickens fed ad libitum and feed-restricted

Feed restriction[a]	Leptin dose (μg/kg body weight/day)	No. of hens in which the respective follicles were identified				
		White		Yellowish, >6–8 mm	Yellow	
		l–4 mm	>4–6 mm		>8–12 m	>12 mm
No (control)	0	6	3	1	1	1
No	256	6	6	6	6	6
Yes	0	6	1	None	None	None
Yes	256	6	3	1	1	None

[a] 82–85% of ad libitum control group.

4. Discussion

The current study is the first to assess the effect of exogenous leptin administered daily during prepubertal development on the timing of reproductive maturity in a non-mammalian species. The chosen doses of leptin (4–256 μg/kg body weight/day) were in the range used in the former experiments [24] and commonly applied for small animals [26,28,29,48–50]. Our results clearly demonstrate that subcutaneous injections of recombinant chicken leptin to immature female chickens of layer strain significantly advanced the onset of puberty, evidenced by age at first oviposition, and that this advancement was associated with attenuation of ovarian apoptosis. This leptin effect was observed both in ad libitum-fed and feed-restricted birds. In birds fed ad libitum, the effect of leptin on the age of attainment of sexual maturity tended to be dose-dependent, although this effect reached significance only in birds receiving the highest dose (256 μg/kg body weight/day). Interestingly, in these birds the stimulatory effect of leptin on the growth rate (4.2–5.9%) was not accompanied by any effect on feed intake and hence indicates that leptin improved utilization of nutrients, confirming the recent study performed in the Japanese quail in which leptin injected in ovo enhanced the growth rate during embryonic and postembryonic development leading to earlier hatching and puberty [31]. Furthermore, whereas single injection of leptin to chickens [24,26–30] resulted in attenuation of feed intake, this is the first report showing that chronic leptin injections lasting several weeks had no effect on feed consumption. Resistance to chronic leptin treatment was shown in rats which are so far the most leptin-sensitive species. Leptin treatment lasting 150 days by using adeno-associated virus encoding rat leptin cDNA caused a temporary decrease in weight gain and feed intake which was gradually abolished after 10 days [51]. It was suggested that waning of central leptin effect was a result of developing leptin resistance. Thus, it is possible that similar effect may also exist in chickens and development of resistance to central feed-attenuating leptin effect was responsible for lack of the decrease in feed consumption, whereas the peripheral effect of leptin influencing ovary was not affected. Our present results raise the question whether body weight per se might be the factor determining the advancement of puberty. At present an ultimate answer cannot be given since the weight-increasing action of leptin during sexual maturation of avian species is quite novel finding. However, in feed-restricted birds leptin treatment did not affect body weight while similarly as in full-fed birds advanced

the onset of puberty by 6 days and puberty occurred at significantly lower body weight than in ad libitum-fed control birds. In contrast, the anti-apoptotic action of leptin was in full-fed and feed-restricted birds consistently related to the effect on puberty. Therefore, these results suggest that the increase in body weight observed in full-fed birds treated with leptin was unrelated to the leptin effect on reproduction. Involvement of leptin in regulating GH axis cannot be excluded as such leptin effect was reported in rodents [52,53], pigs [54] and humans [20]. However, in poultry GH has much less growth promoting effect than in mammals (for review, see [55]) and so far in avian species leptin-GH axis has not been studied.

Sexual maturation is associated with progressive activation of the hypothalamic-pituitary-gonadal axis, which in females culminates in first ovulation (for review, see [56,57]). In chickens, a prepubertal rise in plasma LH occurring 5–3 weeks prior to egg laying [58–61] is suppressed by the increasing concentrations of E2 produced by the ovarian stroma and the theca layer of white follicles 3–1 week before puberty [60,62,63]. Progesterone, produced by the granulosa layer of large, yellow hierarchical follicles, increases sharply just prior to the first ovulation triggering the preovulatory LH surge [59,61,63,64]. Our data show that the timing of these events remains constant when leptin advances the age at first oviposition, even in hens subjected to feed restriction. It is unclear from our data whether injected leptin acted both centrally and/or peripherally to advance folliculogenesis. The presence of leptin receptor mRNA [34–36,65] and leptin receptor protein [66] at all sites of the chicken hypothalamic-pituitary-ovarian axis makes such a possibility feasible. Moreover, the stimulatory effect of leptin on the in vivo E2 and P4 production by the ovary of fasted hens [36] and the in vitro effect of leptin on steroids secretion by the granulosa and the theca layer of chicken ovarian follicles [67] supports the option of a direct action of leptin at the ovary. In mammalian species leptin seems to have both central and peripheral effects on activating and functioning of the hypothalamic-pituitary-ovarian axis since: (i) in vivo and in vitro leptin stimulates the release of LHRH from the hypothalamus, and FSH and LH from the pituitary (for review, see [4,5,68]), (ii) transfer of leptin-receptor gene to the hypothalamus of the obese and infertile female *fa/fa* Zucker rats having missense mutation in leptin receptor stimulates the hypothalamic-pituitary-ovarian axis [69], (iii) leptin and its receptor are expressed in several ovarian cell types, including the granulosa and the theca cells (for review, see [70]), (iv) in vivo and in vitro leptin affects ovarian steroidogenesis and ovulation (for review, see [4,5]), (v) administration of recombinant leptin accelerates puberty by attenuating ovarian apoptosis and enhancement of follicular development [14] and (vi) leptin deficiency in female *ob/ob* mice is associated with impaired folliculogenesis and increased follicular apoptosis [71].

It is well known that the timing of puberty is tightly coupled to the nutritional and metabolic state of animals and undernutrition delays the progression of sexual maturation. In order to elucidate whether leptin administration may overcome the negative influence of undernutrition, as it was previously demonstrated in female rats [15,17], we compared the effect of daily injections of leptin in ad libitum-fed and feed restricted birds. The 15–18% feed restriction significantly delayed the onset of sexual maturity by 7 days, while leptin treatment abolished this delay, restoring the normal timing of puberty. The ovary weight of 106-day-old immature chickens fed ad libitum and receiving leptin was 2.7-fold higher, while of feed-restricted pullets was 2.3-fold lower than the ovary weight of fed ad libitum,

placebo-injected control group. In feed-restricted birds leptin treatment had no effect on the ovary weight on day 106, indicating that at this age exogenous leptin did not rescue the delayed growth of the ovary caused by undernutrition. However, regarding the age of puberty attainment we anticipate that the stimulatory effect of leptin would be found at the later age of birds. Morphological characteristics of the ovaries of 106-day-old pullets showed no significant differences among the examined groups in the weight of ovarian stroma containing cortical follicles <1 mm as well as in the number and weight of 1–4 mm white follicles which were present in the ovaries of all examined birds. In contrast, the presence of other classes of ovarian follicles, i.e. white (>4–6 mm), yellowish (>6–8 mm) and yellow hierarchical (>8 mm) follicles varied considerably among treatment groups and reflected the age at which they subsequently attained sexual maturity. Full-fed birds receiving leptin had yellow follicles entering the hierarchy at 12 days prior to their first oviposition, while feed-restricted birds, destined to egg laying 13 days later than the earliest group, had neither yellow nor yellowish. The birds that subsequently attained sexual maturity at the same time (ad libitum controls and feed-restricted plus leptin) exhibited similar ovarian development at day 106. This result, coupled with the hormonal data, suggests that the effect of leptin on sexual maturation was manifested after a relatively short period of daily injections. The advancement of the onset of puberty induced by exogenous leptin in ad libitum-fed and feed-restricted chickens, in which normal timing of puberty was restored, raises the question whether in birds leptin activates the reproductive axis or serves as a permissive factor whose presence is required but not sufficient, as suggested regarding the role of leptin in regulating puberty in mammalian species [16,68]. To answer this question the measurements of circulating leptin concentrations and the expression of leptin receptor mRNA and protein at the hypothalamic-pituitary-ovarian axis during prepubertal development need to be examined.

We have previously reported that leptin exhibits an anti-apoptotic activity in the ovary of mature chickens subjected to fasting [36]. In the present study, we assessed whether the enhancement of sexual maturation by leptin injections might also be associated with alterations in ovarian apoptosis. Our finding that the number of apoptotic cells in cortical follicles was significantly decreased by leptin treatment, both in fed ad libitum and feed-restricted birds, indicates that this action of leptin on the ovary is independent of nutritional restriction. Furthermore, leptin-dependent reduction in the number of apoptotic cells was unrelated to the specific physiological stage. Feed-restricted hens treated with leptin had the similar ovary weight on day 106 and reached puberty at almost the same time as ad libitum fed control birds not treated with leptin (see Table 2), but on day 106 the anti-apoptotic effect of leptin treatment was evident and comparable to that observed in leptin treated ad libitum fed hens that reached puberty 7 days earlier. Thus, we can conclude that the anti-apoptotic effect is related to leptin and not to the specific physiological state. In white follicles the similar tendency was found but the effect was not significant. Similarly, Almog et al. [14] demonstrated in rats that acceleration of puberty by exogenous leptin is associated with decreased ovarian apoptosis, enhanced folliculogenesis and increased Bcl-2/Bax ratio. Furthermore, Hamm et al. [71] showed that leptin deficiency in *ob*/*ob* mice is associated with increased ovarian apoptosis, impaired folliculogenesis and elevated Fas/Fas ligand expression. Hence, the anti-apoptotic action of leptin in the ovary seems to be a part of a general mechanism responsible for activating and maintaining ovarian

function. Recent studies have revealed that the cellular processes that regulate follicular development are highly conserved among avian and mammalian species (for review, see [72,73]). Interestingly, in other tissues, like mouse fat cells [74] or human bone marrow stromal cells [75] leptin promotes apoptosis, indicating that leptin's effects might be tissue-specific.

Taken as a whole, data from the current study suggest that leptin plays a physiological role in regulating sexual maturation of the domestic hen and that its action is associated with attenuation of ovarian apoptosis and enhancement of folliculogenesis. However, it is unclear whether leptin stimulates follicular development by acting solely at the central level or also at the peripheral level. It is also unknown whether leptin is a critical, permissive, or facilitator factor in its regulation of puberty. Those aspects of leptin actions will be shortly explored by application of leptin mutants, prepared recently in our lab, which act as blockers (competitive inhibitors) of endogenous leptin [76].

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